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Exhibit C

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Abstracts

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Tuesday, July 6, 1993

09.30 - 11.00

Oral Communications

Physiological Coagulation Inhibitors

Sheraton
New York Ballroom

TUESDAY

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STRUCTURAL DISSECTION OF THE ANTITHROMBIN III HEPARIN BINDING SITE: K107 AND K114 ARE NOT DIRECTLY INVOLVED IN HEPARIN BINDING. E. Ersdal-Badju, A. Lu, V. Picard, and S.C. Bock. Dept. of Microbiology & Immunology and The Thrombosis Research Center, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140

Ionic interactions between positively charged amino acids of antithrombin III (ATIII) and negatively charged sulfate and carboxylate groups of heparin are thought to mediate ATIII heparin binding and the associated heparin cofactor activity of ATIII. Based on genetic variant, chemical modification and glycosylation isoform data, a cluster of positively charged residues surrounding helix D was proposed to be the heparin binding site of ATIII (Borg et al., 1988, JCI, 81:1292). Lysines 107 and 114 have been assigned to the binding site based on chemical modification studies with S-DABITC (Chang, 1989, JBC, 264:3111) and TNBS (Liu and Chang, 1987, JBC, 262:17356), respectively. However, in contrast to chemical modification data, molecular modeling of the interaction between antithrombin III and an ATIII-binding pentasaccharide of heparin suggests that K107 and K114 do not interact directly with heparin (Grootenhuis and van Boeckel, 1991, JACS, 113:2743).

Using a baculovirus system, we expressed human antithrombin III K107A/N135A and K114A/N135A variant proteins. The background N135A substitution prevents addition of an oligosaccharide which sterically blocks the heparin binding site, and allows for the production of homogeneous ATIIIs-like molecules with increased heparin affinity. As a control, ATIII R47H, with a known defect in heparin binding (Owen et al., 1987, Blood 69:1275), was also expressed. All variants were functional protease inhibitors as evidenced by the formation SDS-stable complexes with thrombin. Salt gradient elution profiles (pH 7.5) of the K107/N135A and K114/N135A variants from HI-trap heparin columns (Pharmacia) were indistinguishable from that of the parental N135A molecule (1.9 M NaCl), while the R47H variant eluted earlier (0.8-0.9 M NaCl) than expressed wildtype ATIII (1.5 M NaCl). These data indicate that binding of ATIII to heparin does not involve ionic interactions between the side chains of lysines 107 and 114 and negatively charged groups on heparin.

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MECHANISM OF ACCELERATION OF ANTITHROMBIN-PROTEINASE REACTIONS BY HEPARIN WITH LOW ANTITHROMBIN AFFINITY. V. I. Streusand*, I. Björk+, and S. T. Olson*. *Henry Ford Hospital, Detroit, MI, USA and +Swedish University of Agricultural Sciences, Uppsala, Sweden.

The accelerating effect of low-affinity heparin (LAH) lacking the pentasaccharide binding site for antithrombin (AT) on the reactions of AT with thrombin and factor Xa was characterized by binding and stopped-flow kinetic studies at I 0.15, pH 7.4, 25°C. LAH (M_r ~8000) was freed of high-affinity heparin (HAH) bearing the AT-binding sequence by repeated affinity chromatography on matrix-linked AT to a constant specific activity. Complete removal of HAH from LAH (<.001%) was indicated from the lack of exchange of added fluorescein-labelled HAH into the LAH after AT-affinity chromatography. Binding of LAH to AT, detected by protein fluorescence changes or by quantitative affinity chromatography (QAC), indicated a weak binding affinity with an estimated K_D > 100 μM; i.e., ~10^4-fold weaker than the affinity of HAH for AT. In contrast, LAH binding to thrombin, monitored by extrinsic probe fluorescence changes or by QAC, was indistinguishable from HAH binding with a K_D of ~1 μM. Consistent with these differential affinities, the accelerating effect of LAH on the pseudo-first order rate constant (k_{obs}) for AT inhibition of thrombin increased as a function of LAH concentration (up to 10 μM) in parallel with the saturation of thrombin with LAH, but was diminished at higher LAH concentrations in parallel with LAH binding to AT. LAH saturation curves showed increased maximal accelerations with increasing levels of AT, indicating the saturation of a ternary complex with a K_D (20 μM) 150-fold weaker than that with HAH, but a maximum acceleration (6 s^{-1}) similar to that of HAH. Contrasting these results, LAH acceleration of k_{obs} for the AT-factor Xa reaction increased in parallel with the binding of AT to LAH with no decrease up to 100 μM LAH, reaching a maximum acceleration (~80-fold) that was 7-fold less than that of HAH. That this acceleration was not due to contaminating HAH was indicated by the indistinguishable saturation of the accelerating effect when LAH was varied at 10 μM AT or AT was varied at 10 μM LAH. These results indicate common mechanisms for LAH and HAH acceleration of AT-proteinase reactions: a ternary complex bridging mechanism for the acceleration of the AT-thrombin reaction, but a heparin-induced AT conformational change-mechanism for the acceleration of the AT-factor Xa reaction. Moreover, the different accelerating effects of these heparins are primarily due to extreme differences in their ability to bind AT and less to differences in their maximum accelerating effect or ability to induce a conformational change in AT.

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THE MECHANISM OF INACTIVATION OF FACTOR Va BY ACTIVATED PROTEIN C INVOLVES TWO CLEAVAGES OF THE HEAVY CHAIN OF THE COFACTOR: (1) Arg_{505} AND (2) Arg_{306}. Michael Kalafatis and Kenneth G. Mann, Department of Biochemistry, University of Vermont, Burlington Vermont, USA.

Factor Va (FVa) is an important cofactor for the activation of prothrombin. The inactivation of FVa by activated protein C (APC) has been correlated with the cleavage of factor Va heavy chain (FVa_{HC}). Membrane-bound FVa is rapidly and completely inactivated by APC. However, in the absence of phospholipid vesicles (PCPS) and after complete cleavage of FVa by APC, the cofactor retains 30% of its initial cofactor activity. In the absence of PCPS, cleavage occurs at Arg_{505} of FVa_{HC} and results in a M_r=70,000 fragment which contains the NH_2-terminal portion of the FVa_{HC} (residues 1-505), and a COOH-terminal M_r=24,000 fragment (residues 506-713) which is further cleaved by APC at Arg_{306} resulting in a M_r=20,000 fragment and a M_r=4,000 peptide. To understand the process by which APC inactivates FVa, we evaluated its cofactor function using APC inactivated FVa in the presence and absence of PCPS. We found that after 30 min, membrane-bound FVa (200 nM) is completely inactivated by APC (10 nM), whereas in the absence of PCPS, after 2h the cleaved cofactor still retains 60% of its initial cofactor activity. Prolonged incubation of FVa with APC (24h) in the absence of PCPS resulted in a molecule which retained 20% of its initial cofactor activity. The cleavage pattern of the FVa_{HC} observed in the absence of PCPS is different from the cleavage of the cofactor when incubated with APC in the presence of PCPS. The complete loss of activity is correlated with the cleavage of the M_r=70,000 fragment and the appearance of a M_r=40,000 and a M_r=28,000 fragment. A comparison of the NH_2-terminal sequence of the M_r=28,000 fragment with the sequence of bovine factor V indicated a match with residues 307-505 of FVa_{HC} whereas the NH_2-terminal sequence of the M_r=40,000 was identical to the NH_2-terminal sequence of the FVa_{HC} (residues 1-306). No difference was observed in the cleavage pattern of the light chain of the cofactor (FVa_{LC}) by APC in the presence as well as in the absence of PCPS. Thus, a specific inactivating APC cleavage site (Arg_{306}) is exposed upon binding of the cofactor to PCPS. Our data demonstrates that complete inactivation of FVa by APC occurs after two cleavages of the FVa_{HC}: cleavage at Arg_{505} partially inactivates the cofactor, whereas coordinated cleavage at Arg_{505} and Arg_{306} is responsible for the complete inactivation of FVa.

THE MECHANISM OF INACTIVATION OF FACTOR Va BY ACTIVATED PROTEIN C INVOLVES TWO CLEAVAGES OF THE HEAVY CHAIN OF THE COFACTOR: (1) Arg_{505} AND (2) Arg_{306}.

Michael Kalafatis and Kenneth G. Mann, Department of Biochemistry, University of Vermont, Burlington Vermont, USA.

Factor Va (FVa) is an important cofactor for the activation of prothrombin. The inactivation of FVa by activated protein C (APC) has been correlated with the cleavage of factor Va heavy chain (FVa_{HC}). Membrane-bound FVa is rapidly and completely inactivated by APC. However, in the absence of phospholipid vesicles (PCPS) and after complete cleavage of FVa by APC, the cofactor retains 30% of its initial cofactor activity. In the absence of PCPS, cleavage occurs at Arg_{505} of FVa_{HC} and results in a M_r=70,000 fragment which contains the NH_2-terminal portion of the FVa_{HC} (residues 1-505), and a COOH-terminal M_r=24,000 fragment (residues 506-713) which is further cleaved by APC at Arg_{662} resulting in a M_r=20,000 fragment and a M_r=4,000 peptide. To understand the process by which APC inactivates FVa, we evaluated its cofactor function using APC inactivated FVa in the presence and absence of PCPS. We found that after 30 min, membrane-bound FVa (200 nM) is completely inactivated by APC (10 nM), whereas in the absence of PCPS, after 2h the cleaved cofactor still retains 60% of its initial cofactor activity. Prolonged incubation of FVa with APC (24h) in the absence of PCPS resulted in a molecule which retained 20% of its initial cofactor activity. The cleavage pattern of the FVa_{HC} observed in the absence of PCPS is different from the cleavage of the cofactor when incubated with APC in the presence of PCPS. The complete loss of activity is correlated with the cleavage of the M_r=70,000 fragment and the appearance of a M_r=40,000 and a M_r=28,000 fragment. A comparison of the NH_2-terminal sequence of the M_r=28,000 fragment with the sequence of bovine factor V indicated a match with residues 307-505 of FVa_{HC} whereas the NH_2-terminal sequence of the M_r=40,000 was identical to the NH_2-terminal sequence of the FVa_{HC} (residues 1-306). No difference was observed in the cleavage pattern of the light chain of the cofactor (FVa_{LC}) by APC in the presence as well as in the absence of PCPS. Thus, a specific inactivating APC cleavage site (Arg_{306}) is exposed upon binding of the cofactor to PCPS. Our data demonstrates that complete inactivation of FVa by APC occurs after two cleavages of the FVa_{HC}: cleavage at Arg_{505} partially inactivates the cofactor, whereas coordinated cleavage at Arg_{505} and Arg_{306} is responsible for the complete inactivation of FVa.